

# Anti-Transglutaminase Antibody Assay of the Culture Medium of Intestinal Biopsy Specimens Can Improve the Accuracy of Celiac Disease Diagnosis

ANTONIO CARROCCIO,<sup>1\*</sup> LIDIA DI PRIMA,<sup>1</sup> GIUSEPPE PIRRONE,<sup>1</sup> CALOGERO SCALICI,<sup>2</sup> ADA M. FLORENA,<sup>3</sup> MAURIZIO GASPARIN,<sup>4</sup> GIUSEPPE TOLAZZI,<sup>4</sup> ANTONINO GUCCIARDI,<sup>1</sup> CARMELO SCIUMÈ,<sup>5</sup> and GIUSEPPE IACONO<sup>2</sup>

**Background:** We measured anti-transglutaminase (anti-tTG) antibody in the culture medium of intestinal biopsy specimens from patients with suspected celiac disease (CD) and evaluated the relationship between antibody production and severity of intestinal mucosal damage.

**Methods:** We performed diagnostic testing for CD on 273 consecutive patients. In addition to routine histologic evaluation of duodenal biopsy specimens, we assayed anti-tTG antibodies in serum and in the culture medium of duodenal biopsy specimens.

**Results:** CD was diagnosed in 191 of the 273 patients. Sensitivity and specificity of the serum anti-endomysium (EmA) and anti-tTG assays were 83% and 85% and 99% and 95%, respectively, and both had 88% diagnostic accuracy. EmA and anti-tTG assayed in the culture medium had 98% sensitivity, 100% specificity, and 98% diagnostic accuracy (vs serum assays;  $P < 0.0001$ ). Twenty-nine CD patient specimens (16%) were negative for serum anti-tTG and EmA; for 24 of these patients, anti-tTG assay of the culture medium was positive. The CD patients whose biopsy specimens were positive for serum antibodies showed the following intestinal histologies: total villous atrophy, 35%; severe villous atrophy, 25%; mild atrophy, 25%; villi with no atrophy but with increased intraepithelial lymphocytes, 15%. None

of the CD patients whose specimens were negative for serum antibodies showed total or severe villous atrophy; 77% had mild villous atrophy, and 23% had no villous atrophy but had increased intraepithelial lymphocyte counts. Mild villous atrophy was also seen in specimens from ~15% of patients without CD.

**Conclusion:** Anti-tTG assay of the culture medium of biopsy specimens can improve the accuracy of CD diagnosis in patients negative for serum antibodies.

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Celiac disease (CD)<sup>6</sup> is one of the most common chronic diseases among Caucasians, occurring in 1 in 100 to 300 individuals in Europe and the United States (1–4). Diagnosis is based on the histologic findings of hyperplastic villous atrophy while the patient is eating gluten and subsequent full clinical remission after the exclusion of gluten from the diet (5). Because CD patients may have intestinal mucosal lesions less severe than the typical diagnostic “subtotal or total villous atrophy”, various degrees of intestinal damage, including simple mucosal inflammation with minimal villous damage, are now considered as indications of possible CD (6). Consequently, the presence of circulating antibodies, mainly anti-transglutaminase (anti-tTG) and anti-endomysium (EmA) antibodies, on diagnosis and their disappearance when the patient is following a gluten-free diet are considered important data to support the CD diagnosis in cases with mild intestinal lesions. According to the original criteria of the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) (7), a gluten challenge and several

<sup>1</sup> Internal Medicine, <sup>3</sup> Pathology Department, and <sup>5</sup> Surgery Department, University Hospital of Palermo, Palermo, Italy.

<sup>2</sup> Pediatric Gastroenterology, Di Cristina Hospital, Palermo, Italy.

<sup>4</sup> Laboratory R&S Eurospital SpA, Trieste, Italy.

\* Address correspondence to this author at: Internal Medicine, University Hospital of Palermo, via del Vespro 141, 90127 Palermo, Italy. Fax 39-091-6552936; e-mail acarroccio@hotmail.com.

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<sup>6</sup> Nonstandard abbreviations: CD, celiac disease; tTG, transglutaminase; EmA, anti-endomysium; and IEL, intraepithelial lymphocyte.

intestinal biopsies must be performed in patients without a definitive initial CD diagnosis, i.e., who do not have marked intestinal damage or serum anti-tTG and EmA.

We recently showed that assay of the culture medium of intestinal biopsy specimens for EmA antibodies can identify an infiltrative/hyperplastic histologic pattern that occurs in CD patients but is often associated with negative serum EmA assay results (8). This finding suggests that in patients with negative serology but with symptoms consistent with CD, an EmA assay of the culture medium of biopsy samples should be performed to help confirm the diagnosis.

We measured anti-tTG antibodies in the culture medium of intestinal biopsy specimens from patients with suspected CD and assessed the diagnostic value of this assay. The relationship between the severity of the intestinal mucosal damage and the production of anti-tTG antibodies was also evaluated.

### Patients and Methods

This prospective study included 120 children (50 males, 70 females; age range, 7 months to 14 years; median age, 14 months) and 153 adults (54 males, 99 females; age range, 17–80 years; median age, 32 years), enrolled from January 2001 to June 2003, who were consecutive patients undergoing intestinal biopsy for suspected CD at 2 centers: a pediatric gastroenterology clinic and an internal medicine clinic. Inclusion criteria were positivity for serum IgA EmA and/or anti-tTG antibodies or, in patients with negative serum EmA and anti-tTG assay results, loss of duodenal plicae and the presence of mucosal scalloping observed during esophagogastroduodenoscopy performed for any reason, or presence of one or more of the following symptoms without evidence of a disease other than CD after a complete work-up: weight loss or failure to thrive, anemia, chronic diarrhea, or abdominal pain. The diagnostic work-up may also have included routine hematochemical assays, a thyroid study, serum autoantibodies, abdominal ultrasonography and/or computed tomography, colonoscopy, small-intestine barium examination, H<sub>2</sub> breath test, duodenal fluid microbiological evaluation, and bone marrow biopsy.

Patients who had undergone previous histologic evaluation for suspected CD were excluded from the study. All adult patients included in the study were followed as outpatients, whereas the children were hospitalized.

In the patients with positive serum anti-tTG and/or EmA assay results, CD diagnosis was based on the appearance of clinical symptoms and histologic indications of intestinal damage while patients were on a gluten-containing diet and the disappearance of the symptoms and normalization of serum anti-tTG and/or EmA antibodies on a gluten-free diet. In the pediatric and adult patients negative for serum anti-tTG and EmA antibodies, diagnosis was based on clinical symptoms and histologic indications of damage on a gluten-containing diet, the disappearance of the symptoms and normalization of the intestinal histologic findings on a gluten-free

diet, and reappearance of the symptoms and mucosal damage on gluten challenge.

The protocol was approved by the Ethics Committee of the University Hospital of Palermo, and informed consent was obtained from the patients involved in the study or from their parents in the case of the pediatric patients.

### SEROLOGY STUDIES

Serum IgA was measured by ELISA to exclude IgA deficiency. Serum IgA recombinant anti-human tTG antibody concentrations were determined with a commercially available ELISA (human Eu-tTG IgA; Eurospital) (9). Results were expressed as a percentage of the positive control serum. Reference values were taken as <7%, representing a value >2 SD above the mean of 850 healthy individuals. Serum IgA EmA antibodies were assayed with a commercially available indirect immunofluorescence method on monkey esophagus (Anti-endomysium; Eurospital) as described previously (9, 10).

### DUODENAL MUCOSA CULTURE AND ASSAYS FOR IgA EmA AND ANTI-tTG ANTIBODIES IN CULTURE MEDIUM

Six duodenal biopsy samples were obtained from each patient by esophagogastroduodenoscopy. Four samples underwent routine histologic evaluation, and 2 specimens were cultured for 72 h at 37 °C with a commercial reagent set (anti-Endomysium-biopsy; Eurospital), as described previously (8). One sample was cultured in the presence of the 31–43 gliadin peptide (0.1 g/L) and the other without its addition. Culture supernatants were collected and stored at –80 °C until used. IgA EmA antibodies in undiluted supernatants were determined by the same commercial reagent set used for serum EmA antibodies, and the positive samples were titrated with progressive dilutions until they became negative.

The concentrations of the IgA recombinant anti-human tTG antibodies in supernatants were determined in the Eurospital laboratory with a new commercial ELISA (Eu-tTG-biopsy; Eurospital). Recombinant h-tTG antigen diluted in phosphate-buffered saline was used to coat the wells. The purity of the recombinant protein was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The culture medium from the EmA biopsy was diluted 1:5. The conjugate was diluted to obtain reliable absorbances. Absorbance was read in a microplate reader at 450 nm. Anti-tTG values in the supernatants were expressed as absorbance. Reference values were taken as <300, representing a value >2 SD above the mean of 200 healthy individuals. The intraassay CV for the IgA h-tTG autoantibody ELISA on culture medium was 3.2% (n = 20), and the interassay CV was 5.4% (n = 20).

IgA EmA and anti-tTG assays of the culture medium were performed by personnel unaware of the clinical and laboratory data of the patients.

A control test performed in our laboratory on 20 culture media in which IgA EmA and anti-tTG antibodies were first assayed on fresh medium and then 6 months

later after storage at  $-80^{\circ}\text{C}$  showed that storage did not significantly alter the results obtained: EmA results were identical, and the interassay CV for IgA anti-tTG was 7.1% ( $n = 30$ ).

#### INTESTINAL BIOPSY AND HISTOLOGY

Biopsy specimens were obtained from the second duodenal portion during gastroduodenoscopy. Specimens adequate in size were immediately oriented with the aid of a stereomicroscope and subsequently embedded in paraffin (8–10). The slides were stained with hematoxylin and eosin and graded according to the standardized scheme reported by Oberhuber et al. (6). The number of intraepithelial lymphocytes (IELs) per 100 villous epithelial cells was assessed as described by Ferguson and Murray (11); the upper limit of the reference interval in our laboratory is 30 IELs/100 epithelial cells. In all cases, histologic analysis was performed by an examiner unaware of the clinical condition and laboratory test results of the patients.

#### STATISTICAL ANALYSIS

We followed the STARD checklist for studies on the diagnostic accuracy of tests (12). The sensitivity, specificity, and diagnostic accuracy of the methods examined were calculated by standard statistical methods (13). The Fisher exact test was used to compare the sensitivity, specificity, and diagnostic accuracy of the assays. The Spearman correlation coefficient was calculated to evaluate the relationship between anti-tTG values and EmA titers assayed in the culture medium. The  $\chi^2$  test for trend was used to compare the percentages of the different intestinal mucosa lesions in the CD patients with positive serology, in the CD patients with negative serology, and in the non-CD patients.

### Results

#### FINAL DIAGNOSES IN THE STUDY GROUP

None of the patients enrolled refused to undergo intestinal biopsy, and none showed IgA deficiency. Data for the adult and pediatric patients, grouped according to the different inclusion criteria and the final diagnoses, are

shown in Table 1. Histologic findings and the clinical follow-up confirmed that 162 of the 166 patients positive for serum EmA and/or anti-TG antibodies had CD. The other 4 patients (1 positive for EmA and anti-tTG antibodies, and 3 positive only for anti-tTG antibodies) in whom CD diagnosis was excluded showed a normal duodenal histology, with a villi/crypts ratio  $>3.5$  and  $<30$  IELs/100 epithelial cells (range, 12–20).

Among the patients who underwent intestinal histologic evaluation and were negative for serum EmA and tTG antibodies, CD was diagnosed in 13 of 83 adults and 16 of 24 children. Duodenal histologic findings were compatible with CD diagnosis at entry to the study: symptoms disappeared and duodenal histologic findings normalized on a gluten-free diet, and symptoms and mucosal damage reappeared on gluten challenge.

In total, 81 adults and 110 children had a final diagnosis of CD. In the adult patients, 72 had diagnoses other than CD (more than 1 diagnosis was present in each patient): sideropenic anemia (66 cases), irritable bowel syndrome (58 cases), peptic ulcers (30 cases), multiple food hypersensitivity (19 cases), systemic erythematous lupus (3 cases), rheumatoid arthritis (1 case), intestinal giardiasis (2 cases), autoimmune enteropathy (1 case), and refractory sprue (1 case). The 10 children without CD had the following final diagnoses: cow's milk protein intolerance (5 cases), multiple food intolerance (3 cases), insulin-dependent diabetes mellitus (1 case), and intestinal giardiasis (1 case).

#### RESULTS OF IgA EmA AND ANTI-tTG ASSAYS OF THE CULTURE MEDIUM

Assays for IgA EmA and anti-tTG antibodies in the culture medium of the intestinal biopsy specimens showed an identical pattern: all cases positive for EmA antibodies were also positive for anti-tTG antibodies, and all cases negative for EmA antibodies were also negative for anti-tTG antibodies. The results were not modified when the 31–43 peptide was added to the culture medium; there was no difference between the assays performed on the culture medium with or without added peptide. As shown in Fig. 1, we found a highly significant

**Table 1. Number of adult and pediatric patients divided according to the different inclusion criteria and the final diagnoses.<sup>a</sup>**

	Final diagnosis of CD, n (%)	Final diagnosis other than CD, n (%)
<b>Adults</b>		
Patients with positive serum EmA and/or tTG ( $n = 70$ )	68 (97)	2 (3)
Patients with negative serum EmA and tTG ( $n = 83$ )	13 (16)	70 (84)
Total ( $n = 153$ )	81 (53)	72 (47)
<b>Children</b>		
Patients with positive serum EmA and/or tTG ( $n = 96$ )	94 (98)	2 (2)
Patients with negative serum EmA and tTG ( $n = 24$ )	16 (67)	8 (33)
Total ( $n = 120$ )	110 (92)	10 (8)

<sup>a</sup> The patients negative for serum EmA and/or anti-tTG were included in the study because they showed symptoms or esophagoduodenoscopic findings suggesting a diagnosis of CD.

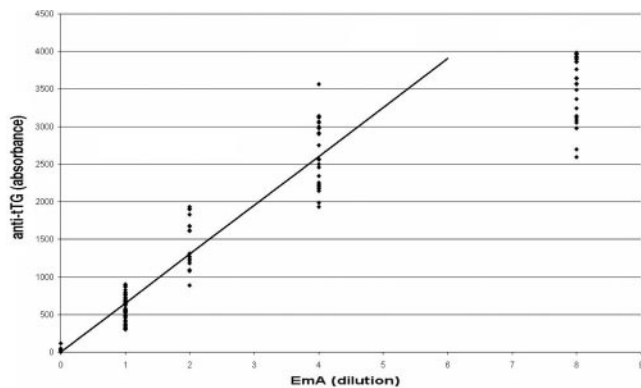


Fig. 1. Correlation between EmA titer (expressed as dilution coefficient) and anti-tTG antibody concentration (expressed as absorbance) assayed in the culture medium of the intestinal biopsy specimens from 191 consecutive patients who underwent intestinal biopsy and had a final diagnosis of CD.

Equation for the line:  $y = 650.64x$  ( $R^2 = 0.9058$ )

statistical correlation between the EmA titer and the absorbance obtained in the anti-tTG assay of the culture medium of the intestinal biopsies of the CD patients, without added 31–43 gliadin peptide [Spearman correlation coefficient ( $R^2$ ) = 0.905;  $P < 0.0001$ ].

**BEHAVIOR OF EmA AND ANTI-tTG ANTIBODIES IN ASSAYS OF THE CULTURE MEDIUM OF INTESTINAL BIOPSY SPECIMENS FROM CD AND NON-CD PATIENTS**  
All CD patients, both children and adults, positive for serum EmA and/or anti-tTG antibodies were also positive for EmA and anti-tTG antibodies in the culture medium. Furthermore, in 24 of the 29 CD patients negative for serum EmA and anti-tTG antibodies (all 16 children and 8 of the 13 adult patients), both of these antibodies were positive in the culture medium of the intestinal biopsy specimens. None of the patients with a final diagnosis other than CD had positive EmA and anti-tTG assay results for the culture medium, including the 2 adults (1 with systemic erythematous lupus and 1 with rheumatoid arthritis) and 2 children (with intestinal giardiasis and insulin-dependent diabetes mellitus, respectively) whose results were false positive for serum EmA and/or anti-tTG antibodies. A cross-tabulation of the results for serum EmA and anti-tTG antibodies and anti-tTG antibodies in the culture medium of the intestinal biopsies, according to final diagnosis, is shown in Table 2.

#### DIAGNOSTIC ACCURACY OF EmA AND ANTI-tTG ASSAYS OF SERUM AND CULTURE MEDIUM

Shown in Table 3 are the sensitivity, specificity, and diagnostic accuracy for diagnosing CD based on the results of the assays for serum EmA and serum anti-tTG antibodies, together with assays for EmA and anti-tTG in the culture medium of the intestinal biopsy specimens. Although the specificities of the serum and culture me-

**Table 2. Cross-tabulation of the serum EmA, serum anti-tTG, and culture medium anti-tTG assay results in patients with suspected CD, divided according to the final diagnoses.**

	CD, n	Non-CD, n	Total, n
Serum EmA			
Positive	159	1	160
Negative	32	81	113
Total	191	82	273
Serum anti-tTG			
Positive	162	4	166
Negative	29	78	107
Total	191	82	273
Culture medium anti-tTG			
Positive	186	0	186
Negative	5	82	87
Total	191	82	273

dium assays did not differ, the sensitivity and diagnostic accuracy were significantly higher for the culture medium assays ( $P < 0.0001$  for both, Fisher exact test).

#### RELATIONSHIP BETWEEN SERUM EmA AND ANTI-tTG RESULTS AND SEVERITY OF DAMAGE TO INTESTINAL MUCOSA

The intestinal histologic findings for the 273 consecutive patients who underwent intestinal biopsy for suspected CD, according to the final diagnoses and the behavior of the serum EmA and anti-tTG antibodies, are presented in Fig. 2.

CD patients with positive serum EmA and/or anti-tTG assay results clearly showed more severe intestinal mucosal lesions; severe or total villous atrophy (grade 3b or 3c) was seen in 25% and 35% of this group, respectively (vs seronegative CD patients,  $P < 0.001$ ; vs non-CD patients,  $P < 0.0001$ ). Mild villous atrophy (grade 3a) was seen in 25% of these patients, and 15% of them showed only an increase in the IEL number without a significant reduction in villi height (grade 2 lesions). Anti-tTG antibodies assayed in the culture medium were positive in all of these patients. Intestinal mucosal damage was less severe in the 29 CD patients with negative serum EmA and anti-tTG antibodies: none of them showed total villous atrophy, 77% had mild villous atrophy (grade 3a), and 23% had only an increase in IEL number without villous damage (grade 2 lesion). Anti-tTG antibodies assayed in the culture medium were positive in 24 of these patients, as only 5 patients with grade 2 lesions did not show anti-tTG positivity.

In the patients without CD, mild villous atrophy was seen in 11 cases: 5 children [multiple food hypersensitivity (3 cases), cow's milk protein intolerance (1 case), intestinal giardiasis (1 case)] and 6 adults [multiple food intolerance (4 cases), systemic erythematous lupus (1 case), and autoimmune enteropathy (1 case)]. Another adult patient with refractory sprue, who was never posi-



**Table 3. Sensitivity, specificity, and diagnostic accuracy for CD diagnosis of serum EmA and anti-tTG assays and EmA and anti-tTG assays of intestinal biopsy specimen culture medium.<sup>a</sup>**

	True positives, <sup>a</sup> n	Sensitivity, <sup>b</sup> %	True negatives, <sup>c</sup> n	Specificity, <sup>b</sup> %	Diagnostic accuracy, <sup>b</sup> %
Serum					
EmA assay	159	83 (78–88)	81	99 (96–100)	88 (83–91)
Anti-tTG assay	162	85 (79–89)	78	95 (90–99)	88 (83–91)
Culture medium					
EmA assay	186	98 (95–100)	82	100 (99–100)	98 (96–99)
Anti-tTG assay	186	98 (95–100)	82	100 (99–100)	98 (96–99)

<sup>a</sup> Total number of final CD diagnoses, n = 191.<sup>b</sup> Values in parentheses are the 95% confidence interval.<sup>c</sup> Total number of final diagnoses other than CD, n = 82.

tive for serum EmA, anti-tTG, or anti-enterocyte antibodies, showed severe villous atrophy. In 58% of this patient group, we observed slight intestinal mucosal lesions, characterized by normal villi but a high IEL number (40–65 IELs/100 enterocytes). None of the non-CD patients had a positive result for anti-tTG antibodies in the culture medium.

### Discussion

The serologic tests for CD have 3 main roles in clinical practice: (a) identifying individuals who require an intestinal biopsy examination to diagnose the disease; (b) supporting the diagnosis in individuals with characteristic histologic features of CD; and (c) monitoring dietary compliance. The first aspect seems to be fully confirmed by recent reviews showing that serum IgA EmA antibodies have 90%–100% sensitivity and 95%–100% specificity and that IgA anti-tTG antibodies show very similar values (14, 15), mainly obtained with the second-generation, human anti-tTG assays (9, 16). However, the presence of positive serum antibodies has been shown to correlate with the degree of villous atrophy, and CD patients with

less severe histologic damage can be seronegative for CD (17–20). It has been demonstrated previously that the culture medium of intestinal biopsy specimens from some of these CD patients showed positive EmA antibodies, and this positivity can be useful for CD diagnosis in patients with minimal intestinal histologic lesions (8, 21).

As tTG is known to be the sole (22) or main antigen of EmA antibodies, in this study we aimed to assay anti-tTG in the culture medium of intestinal biopsies from patients with suspected CD. By applying standard criteria (5, 7) or more rigorous criteria for cases with negative serum antibodies, we made 191 new CD diagnoses. Approximately 15% of these (29 of 191 patients) were seronegative, a frequency similar to that reported previously by others (23). Interestingly, 24 of the 29 seronegative CD patients were positive for EmA and anti-tTG antibodies assayed in the culture medium of the intestinal biopsy specimens. This result suggests that the assay for biopsy specimen culture medium was more sensitive than the serum assay. This higher sensitivity could be related to the previously reported finding that EmA (24) and anti-tTG (25) antibodies are produced by the intestinal mucosa in CD patients. As a consequence, we hypothesize that for cases with low antibody production, antibodies would not be found in the serum but only in the primary site of production. Therefore, the higher sensitivity of the culture medium assay is associated with a higher diagnostic accuracy. Furthermore, the 4 patients with false-positive serum EmA or anti-tTG results had negative results in the culture medium assay. The finding that 2 of these patients were suffering from autoimmune diseases provides evidence that serum anti-tTG results can be false positive in these cases (9) and that the site of antibody production may be other than intestinal (26).

Our results demonstrate the possibility of assaying anti-tTG antibodies in the culture medium of intestinal biopsy specimens and the concordance of these assay results with the EmA assay, an important finding because use of the EmA assay can be limited by increased costs, time-consuming protocols, and above all, by the susceptibility of the EmA assay to subjective interpretation, which may lead to unacceptable variations between laboratories.

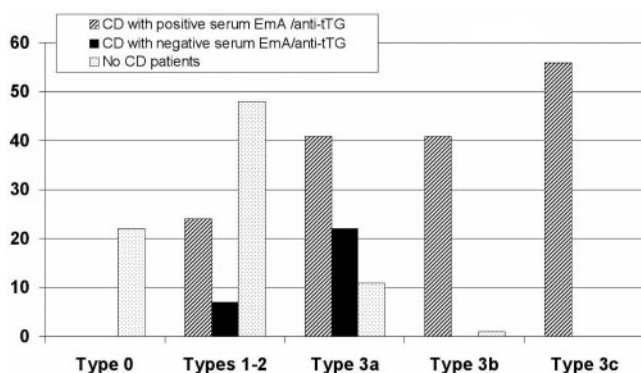


Fig. 2. Intestinal histologic findings in the CD patients with positive serum antibodies (▨; n = 162), in those with negative serum EmA and anti-tTG antibodies (■; n = 29), and in the 82 patients with diseases other than CD (□).

Severity of intestinal damage was classified according to a modified version of the system used by Oberhuber et al. (6) as follows: type 0, normal mucosa; type 1, normal villi and crypt architecture, with >30 IELs/100 epithelial cells; type 2, normal villi with crypt hyperplasia and >30 IELs/100 epithelial cells; type 3a, mild villous flattening; type 3b, marked villous flattening; type 3c, total villous flattening.

Assays of the culture medium of biopsy specimens have practical clinical relevance because their results can facilitate correct diagnosis. Our data show that the "simple" intestinal histologic evaluation does not allow definitive CD diagnosis in cases in which serum antibodies are not detected. In our study group, 23% of the seronegative CD patients showed only increases in IEL numbers and crypt hypertrophy without villous damage. Mild villous atrophy was also seen in ~15% of symptomatic patients without CD, and 58% patients of these showed slight intestinal mucosal lesions (normal villi but high IEL number). According to the standard diagnostic criteria for CD, all of these patients would have to undergo several intestinal biopsies during different dietary treatment regimens, including a gluten challenge. Because of the diagnostic accuracy of the assays performed on the culture medium, the addition to the standard endoscopic procedure of collection of an additional biopsy sample for in vitro culturing of intestinal mucosa and subsequent anti-tTG assay on the culture medium can simplify the diagnosis of CD and improve its accuracy. In clinical practice we suggest use of the biopsy specimen antibody assay method in all cases of suspected CD, even those negative for serum anti-tTG or EmA antibodies, and in all other suspected cases showing conflicting laboratory and histologic data.

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